

Herpesvirus type 1–8 in BAL fluid from HIV-1-infected patients with suspected pneumonia and from healthy individuals

B. Tarp*, S. Jensen-Fangel*, R. Dahl[#], N. Obel*

Herpesvirus type 1–8 in BAL fluid from HIV-1-infected patients with suspected pneumonia and from healthy individuals. B. Tarp, S. Jensen-Fangel, R. Dahl, N. Obel. ©ERS Journals Ltd 2001.

ABSTRACT: Pneumonia is still a major problem in human immunodeficiency virus (HIV)-infected patients, and despite extensive investigation the aetiology remains unknown in many cases. The prevalence of the eight human herpesviruses was determined by polymerase chain reaction in 91 samples of bronchoalveolar lavage (BAL) fluid from 72 HIV-infected patients with 91 episodes of suspected pneumonia. The presence of herpesviruses was related to clinical and immunological findings and the prevalence of herpesviruses in HIV-infected patients was compared with the prevalence in BAL fluid from 50 healthy, immunocompetent individuals.

Epstein-Barr virus, cytomegalovirus and human herpesvirus-8 (HHV8) were found in 5.5%, 36%, and 5.5% of BAL fluid samples from HIV-infected patients. No herpesviruses were detectable in BAL fluid from healthy, immunocompetent individuals. The herpesviruses occurred mainly in patients with CD4⁺ counts <200 × 10⁶ L⁻¹. All patients with herpesviruses recovered without specific antiviral treatment. Two patients with HHV8 had the diagnosis of Kaposi's sarcoma.

It is concluded that cytomegalovirus, Epstein-Barr virus, and human herpesvirus-8 are frequently present in bronchoalveolar lavage fluid from severely immunocompromised human immunodeficiency virus-infected patients with pulmonary symptoms. In bronchoalveolar lavage fluid from healthy, immunocompetent individuals, herpesviruses are absent. Apart from human herpesvirus-8, the present results indicate that the herpesviruses do not play a serious pathogenic role in the development of pulmonary symptoms in human immunodeficiency virus-infected patients.

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*Dept of Infectious Diseases, Marselisborg Hospital, Aarhus University Hospital, and [#]Dept of Respiratory Diseases, Aarhus University Hospital, Aarhus C, Denmark.

Correspondence: B. Tarp, Dept of Infectious Diseases, Marselisborg Hospital, Aarhus University Hospital, DK-8000 Aarhus C, Denmark.
Fax: 45 89491800

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Pulmonary infection is still one of the major causes of morbidity and mortality in the late stages of human immunodeficiency virus-1 (HIV)-infection. In spite of strong evidence of pulmonary infection and use of very extensive microbiological programmes, no causative agent is detectable in a substantial proportion of bronchoalveolar lavage (BAL) fluid samples from HIV-infected/acquired immune deficiency syndrome (AIDS) patients [1]. This may be due to infection with agents which are not routinely investigated, e.g. herpesviruses other than cytomegalovirus (CMV).

CMV has been detected in up to 72% of BAL fluid samples from HIV-infected patients with pulmonary symptoms [2], but its role as a primary pulmonary pathogen has been questioned [3–5]. In 1994 CHANG *et al.* [6] demonstrated human herpesvirus-8 (HHV8) in elements of Kaposi's sarcoma (KS), and the first report of an association of HHV8 with pulmonary KS was made in 1995 by HOWARD *et al.* [7]. The number of studies concerning the prevalence and significance of the remaining six human herpesviruses, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), Epstein-Barr virus (EBV), varicella zoster virus (VZV) and, human herpesvirus-6 and -7 (HHV6 and HHV7), is very limited.

The purpose of this study was to use polymerase chain reaction (PCR): 1) to determine the prevalence of the eight human herpesviruses in BAL-fluids from HIV-infected patients with suspected pneumonia; 2) to relate the presence of herpesvirus deoxyribonucleic acid (DNA) to clinical and immunological findings; and 3) to compare the prevalence of herpesvirus DNA in BAL fluid from HIV-infected patients with pneumonia with the prevalence in healthy, immunocompetent individuals.

Materials and methods

Samples from human immunodeficiency virus infected patients

BAL fluid samples from HIV-positive patients undergoing fiberoptic bronchoscopy for suspected pneumonia between January 1992–August 1998 at the Dept of Infectious Diseases, Aarhus University Hospital, Aarhus, Denmark were included consecutively. Immediately after bronchoscopy BAL fluid was investigated for bacteria and *Pneumocystis carinii*. The

residual BAL fluid was archived and used retrospectively for herpesvirus PCR.

Pneumonia was suspected due to pulmonary infiltrate(s) on the chest radiograph and/or cough and/or reduced arterial oxygen tension at rest ($P_{a,O_2} \leq 10$ kPa). Ninety-one BAL fluid samples representing 91 episodes of pneumonia were obtained from 72 patients. Fifty-eight patients underwent BAL once, nine patients twice and five patients three times. At least 4.5 months passed between two episodes of pneumonia for the patients included more than once. The median age of the patients was 36 yrs (range 23–71). The median CD4+ count was $45 \times 10^6 L^{-1}$ (range $0-1,194 \times 10^6 L^{-1}$). Thirteen of the 72 patients died within 6 months of the bronchoscopy. The other patient characteristics are presented in table 1.

Samples from immunocompetent individuals

BAL fluid from 50 healthy, immunocompetent individuals undergoing consecutive fiberoptic bronchoscopy in connection with another project between January 1991–December 1997 at the Dept of Respiratory Diseases, Aarhus University Hospital, Aarhus, Denmark, was studied.

Bronchoscopy was performed only once in each individual. Forty-six (92%) were males and four (8%) were females. The median age of these individuals was 31 yrs (range 19–65).

Bronchoalveolar lavage

In both of the study populations BAL was performed in accordance with standard techniques by instillation of three aliquots of 60 mL 0.9% sodium chloride, preheated to 37°C, into the radiographically abnormal region of the lung. In the healthy,

immunocompetent patients and in HIV-infected patients with diffuse or no infiltrates, lavage was performed in the middle lobe and/or lingula. No transbronchial biopsies were obtained. BAL fluid was stored immediately at $-80^\circ C$ until analysed.

Sample preparation and polymerase chain reaction amplification of human herpesviruses

DNA was extracted from 300 μL of BAL fluid according to the manufacturers description for Pure-gene® DNA Isolation Kit (Gentra Systems Inc., Minneapolis, MN, USA). Water was extracted following every third sample. The integrity of the extracted DNA was confirmed by PCR for the gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) [8]. In order to control for nonspecific inhibition in the samples which were being negative to PCR-amplification, 1 μL of a PCR-negative sample was mixed with 1 μL of a PCR-positive sample and PCR repeated.

PCR was performed for each of the eight herpesviruses, HSV-1 and -2, VZV, EBV, CMV, HHV6, HHV7, and HHV8 as previously described [9].

A 10 μL aliquot of the PCR product was analysed by electrophoresis in a 2% agarose gel containing 0.5 $\mu g \cdot mL^{-1}$ ethidium bromide and visualized under ultraviolet (UV) light. Assays with suboptimal sensitivity of the positive controls were repeated. With all PCR-positive samples, DNA re-extraction was performed on another aliquot of the original BAL fluid, to exclude cross-contamination. If consecutive samples in the PCR run were positive, they were repeated in separate assays. DNA extraction, mixing of PCR reactions and amplification were performed in three separate laboratories. The detection limits of the PCR assays were <10 genome copies.

Other microbiological examinations

Cultures for bacteria in BAL fluid were performed at the Dept of Clinical Microbiology, Aarhus University Hospital, Aarhus, Denmark. Cultures for mycobacteria were performed at Statens Serum Institute, Copenhagen, Denmark. *P. carinii* was detected by use of immunofluorescence or Gomori methinamine silver stain at the Laboratory of Parasitology, and at the Dept of Pathology, Aarhus University Hospital, Aarhus, Denmark.

BAL fluid from the healthy, immunocompetent individuals was not examined for bacteria or *P. carinii*.

Statistical analysis

Each episode was tested as if it represented a new patient. Univariate analysis was evaluated by odds ratios and Chi-squared tests. The level of significance was 5%.

Ethics

The study was approved by the local ethics committee.

Table 1.—Characteristics of patients

Characteristics	Patients n (%)
Sex	
Male	68 (74.7)
Female	23 (25.3)
Mode of infection	
Homo-/bisexual	47 (51.6)
Heterosexual	32 (35.2)
Injecting drug use	5 (5.5)
Haemophilia	1 (1.4)
Other/unknown	6 (6.6)
CD4+ count $<200 \times 10^6 \cdot L^{-1}$	72 (79.1)
AIDS diagnosis*	43 (47.3)
Kaposi's sarcoma*	8 (8.8)
Antiretroviral treatment*	41 (45.1)
Acyclovir*	8 (8.8)
Pulmonary infiltrate(s)	56 (61.5)
Cough	83 (91.2)
Reduced oxygen tension at rest ≤ 10 kPa	58 (63.7)

Each episode of pneumonia is treated separately and figures relate to 91 episodes in 72 individuals. AIDS: acquired immune deficiency syndrome. *: Meeting the criteria for diagnosis or receiving treatment at the time of bronchoscopy.

Results

Detection of human herpesvirus type 1–8

By PCR, herpesviruses were detected in 40 (44%) of the BAL fluid samples from the HIV-infected patients; EBV in five (5.5%), CMV in 33 (36%), and HHV8 in five (5.5%). HSV-1 and -2, VZV, HHV6, and HHV7 were not detected. A final microbiological diagnosis was obtained in 44 of the samples. In 24 of these, herpesviruses were detected simultaneously with other micro-organisms (table 2), in 16 the herpesviruses were the only micro-organisms detected, and in 31 (34%) no micro-organisms were found.

The three herpesviruses, EBV, CMV, and HHV8 occurred with a higher frequency in patients with CD4+ counts $<200 \times 10^6 \text{ L}^{-1}$ (39 of 72 patients *versus* 4/19 patients ($p < 0.043$)). There was no association between demonstrable herpesvirus and known HIV-infection for >5 yrs, death within 6 months of BAL, presence of the criteria for the diagnosis of AIDS or receiving antiretroviral treatment at the time of bronchoscopy. Eight patients were treated with acyclovir at the time of bronchoscopy. Seven of these had CD4+ counts $<200 \times 10^6 \text{ L}^{-1}$, but herpesvirus was detected in only one patient. None of the patients with herpesvirus(es) in the BAL fluid had signs of stomatitis. None of the eight human herpesviruses were detected in BAL fluid from the healthy, immunocompetent individuals.

None of the BAL fluid samples from the HIV-infected patients or the healthy, immunocompetent individuals showed inhibition in the PCR.

Discussion

The three herpesviruses EBV, CMV, and HHV8 were found to be highly prevalent by PCR, occurring in 44% of BAL fluid samples from HIV-infected patients with suspected pneumonia, while none of the herpesviruses were detectable in BAL fluid from healthy, immunocompetent individuals.

The presence of herpesviruses in patients with low CD4+ counts corresponds with other studies, in which CMV and HHV8 in particular have been shown to occur with higher frequencies in severely immunocompromised patients, while herpesviruses like HHV6 and HHV7 are seen mainly in patients with high

CD4+ counts or in individuals not infected with HIV [10, 11].

In HIV-infected patients, CMV infection/reactivation mainly manifests as retinitis and/or gastrointestinal disease (oesophagitis, colitis, gastritis and hepatitis), and it is reported that $>90\%$ of patients with AIDS will develop CMV infection during the course of their illness [12]. CMV was found in 33 (36%) BAL fluid samples. This high prevalence is in accordance with previous studies in which CMV has been detected in up to 72% of BAL fluid samples from HIV-infected patients with pulmonary symptoms [2]. From most studies it has been concluded that CMV is not the primary pathogen in the pathogenesis of pneumonia [13, 14]. Thus, in contrast to patients with the iatrogenically-induced immunosuppression (post-transplantation), CMV pneumonitis is rare in HIV-infected patients [15]. In a few studies, however, CMV has been reported to be the primary pulmonary pathogen in HIV-infected patients [3, 5]. HANSEN *et al.* [4] have suggested that the detection of CMV by PCR or culture in HIV-infected patients with pulmonary symptoms, can identify patients at high risk for later development of CMV disease. It is unlikely that CMV was the primary pathogen of the respiratory symptoms in the cohort, as the 33 patients with CMV recovered without specific anti-CMV treatment. Furthermore, no correlation was found between the presence of CMV and death within 6 months.

HHV8 was identified in 1994, and since then, several studies have implicated HHV8 as the causative agent of KS [6, 7, 16]. HHV8 was found in BAL fluid samples from five Danish HIV-infected patients. Two of these fulfilled the clinical criteria for KS at the time of bronchoscopy. None of the remaining three patients, who were Caucasians born in Denmark, developed clinical signs of KS before they died. However, magnetic resonance imaging or computed tomography of the thorax was not performed. As it has previously been demonstrated that HHV8 DNA is detectable in peripheral blood mononuclear cells before the development of KS [17], HHV8 DNA in BAL fluid may indicate the presence of subclinical KS.

Respiratory symptoms and chronic interstitial lung disease due to EBV-infection have been reported in immunocompetent individuals, including children [18], but relatively few studies are available concerning EBV and pulmonary disease in patients with AIDS.

Table 2. – Final clinical diagnoses following bronchoscopy related to the detection of herpesviruses

Clinical diagnosis	Herpesviruses						Total
	EBV	CMV	HHV8	EBV+CMV	HHV8+CMV	No HV	
<i>Streptococcus pneumoniae</i> pneumonia		6	1			6	13
<i>Pneumocystis carinii</i> pneumonia		5				4	9
<i>Mycobacterium tuberculosis</i> pneumonia		3				4	7
<i>Mycobacterium pneumoniae</i> pneumonia	2	1					3
<i>Haemophilus influenzae</i> pneumonia		2	3		1	6	12
No clinical diagnosis	1	13		2		31	47
Total	3	30	4	2	1	51	91

EBV: Epstein-Barr virus; CMV: cytomegalovirus; HHV8: human herpesvirus-8; HV: herpesviruses.

EBV seems to be related to the development of AIDS-associated nonHodgkin's lymphoma (NHL) [19]. STOPYRA *et al.* [20] concluded that primary infection or reactivation of EBV is probably an under-reported cause of morbidity and mortality in AIDS patients. In the present study, EBV and CMV occurred together in two BAL fluid samples and EBV alone in one, and they were the only micro-organisms demonstrable in these three fluids. These patients had no radiographic infiltrates, but they had fever and reduced P_{a,O_2} (≤ 10 kPa). The three patients recovered without treatment. The remaining two patients with EBV recovered after treatment for their concomitant bacterial infection (*Mycoplasma pneumoniae*).

A low prevalence of herpesviruses in patients receiving prophylactic acyclovir were found. This finding is not in accordance with a study by LUCHT *et al.* [11]. However, in both studies the number of patients receiving acyclovir is too small for definitive conclusions.

HSV-1 and -2, VZV, HHV6, and HHV7 were not demonstrated, either in the HIV-infected or the immunocompetent individuals. HSV-1 has previously been found in BAL fluid from immunocompromised patients (40% were AIDS patients) and in BAL fluid from patients with complicated respiratory tract infections [21, 22]. The role of HSV-1 recovery in BAL fluid is still unclear, as it is not possible to distinguish between active disease and asymptomatic shedding of the virus. The role of HHV6 and HHV7 in HIV-infection is unknown. Although HHV6 and HHV7 are seen mainly in patients with high CD4+ counts, or in individuals not infected with HIV [10, 11], it has been suggested that simultaneous infection with HIV and HHV6 may contribute to enhanced immunosuppression [23]. The higher prevalence of HHV6 and HHV7 in immunocompetent individuals may be explained by the fact that the CD4+ cells are the main site for active viral replication of these viruses [24]. VZV has only been reported once in a BAL fluid sample, from an HIV-1 infected adult patient with secondary varicella pneumonia [25].

Asymptomatic shedding in saliva of HSV-1, EBV, CMV, HHV6, and HHV7 in healthy, immunocompetent individuals is known to occur [11, 26], but in the present study, these viruses were not found in the cell-associated fluid obtained at lavage.

To the best of the authors' knowledge, this is the first study investigating BAL fluid for all of the eight human herpesviruses in HIV-infected patients, as well as in healthy, immunocompetent individuals. It is concluded, that the human herpesviruses CMV, EBV, and HHV8 are frequently present in BAL fluid from HIV-infected patients with pulmonary symptoms, and that they occur with significantly increased frequencies in severely immunocompromised patients. In BAL fluid from healthy, immunocompetent individuals, the herpesviruses are absent.

Apart from human herpesvirus-8, the results indicate that the herpesviruses do not play a serious pathogenic role in the development of pulmonary symptoms in human immunodeficiency virus-infected patients.

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References

1. Tarp B, Jensen JS, Østergaard L, Andersen PL. Search for agents causing atypical pneumonia in HIV-positive patients by inhibitor-controlled PCR assays. *Eur Respir J* 1999; 13: 175–179.
2. Mann M, Shelhamer JH, Masur H, *et al.* Lack of clinical utility of bronchoalveolar lavage cultures for cytomegalovirus in HIV infection. *Am J Respir Crit Care Med* 1997; 155: 1723–1728.
3. Heurlin N, Brattström C, Lönnqvist B, Westman L, Lidman C, Andersson J. Aetiology of pulmonary diseases in immunocompromised patients. *Eur Respir J* 1991; 4: 10–18.
4. Hansen KK, Vestbo J, Benfield T, Lundgren JD, Mathiesen LR. Rapid detection of cytomegalovirus in bronchoalveolar lavage fluid and serum samples by polymerase chain reaction: correlation of virus isolation and clinical outcome for patients with human immunodeficiency virus infection. *Clin Infect Dis* 1997; 24: 878–883.
5. Waxman AB, Goldie SJ, Brett-Smith H, Matthay A. Cytomegalovirus as a primary pulmonary pathogen in AIDS. *Chest* 1997; 111: 128–134.
6. Chang Y, Cesarman E, Pessin MS, *et al.* Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 1994; 266: 1865–1869.
7. Howard M, Brink N, Miller R, Tedder R. Association of human herpes virus with pulmonary Kaposi's sarcoma. *Lancet* 1995; 346: 712.
8. Kelsen J, Tarp B, Obel N. Absence of human herpesvirus 8 in semen from healthy Danish donors. *Hum Reprod* 1999; 14: 2274–2276.
9. Tarp B, Kelsen J, Nielsen LP, Vinther B, Obel N. Herpesvirus type 1–8 in sinus aspirates from HIV-infected patients and immunocompetent individuals. *Rhinology* 2001; 39: 167–171.
10. Fabio G, Knight SN, Kidd IM, *et al.* Prospective study of human herpesvirus 6, human herpesvirus 7, and cytomegalovirus infections in human immunodeficiency virus-positive patients. *J Clin Microbiol* 1997; 35: 2657–2659.
11. Lucht E, Brytting M, Bjerregaard L, Julander I, Linde A. Shedding of cytomegalovirus and herpesvirus 6, 7, and 8 in saliva of human immunodeficiency virus type 1-infected patients and healthy controls. *Clin Infect Dis* 1998; 27: 137–141.
12. Drew WL. Cytomegalovirus infection in patients with AIDS. *Clin Infect Dis* 1992; 14: 608–615.
13. Miles PR, Baughman RP, Linnemann CC. Cytomegalovirus in bronchoalveolar lavage fluid of patients with AIDS. *Chest* 1990; 97: 1072–1076.
14. Østergaard L, Tarp B, Jensen BN, *et al.* Semi-nested polymerase chain reaction for detection of *Pneumocystis carinii*: Implications for diagnosis, prevalence and predictive parameters. *Immun Infect Dis* 1995; 5: 59–66.

15. Bower M, Barton SE, Nelson MR, *et al.* The significance of the detection of cytomegalovirus in the bronchoalveolar lavage fluid in AIDS patients with pneumonia. *AIDS* 1990; 4: 317–320.
16. Moore PS, Chang Y. Detection of herpes-like DNA sequences in Kaposi's sarcoma patients with and without HIV infection. *N Engl J Med* 1995; 332: 1181–1185.
17. Whitby D, Howard MR, Tenant-Flowers M, *et al.* Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. *Lancet* 1995; 346: 799–802.
18. Pflieger A, Eber E, Popper H, Zach MS. Chronic interstitial lung disease due to Epstein-Barr virus infection in two infants. *Eur Respir J* 2000; 15: 803–806.
19. Pomilla PV, Morris AB, Laworek A. Sinonasal non-Hodgkin's lymphoma in patients infected with human immunodeficiency virus: report of three cases and review. *Clin Infect Dis* 1995; 21: 137–149.
20. Stopyra GA, Multhaupt HA, Alexa L, Husson M, Stern J, Warhol MJ. Epstein-Barr virus-associated adult respiratory distress syndrome in a patient with AIDS: a case report and review. *Mod Pathol* 1999; 12: 984–989.
21. Prellner T, Flamholz L, Haidl S, Lindholm K, Widell A. Herpes simplex virus – The most frequently isolated pathogen in the lungs of patients with severe respiratory distress. *Scand J Infect Dis* 1992; 24: 283–292.
22. Connolly MG, Baughman RP, Dohn MN, Linnemann CC. Recovery of viruses other than cytomegalovirus from bronchoalveolar lavage fluid. *Chest* 1994; 105: 1775–1781.
23. Ablashi DV, Marsh S, Kaplan M, Whitman JE Jr, Pearson GR. HHV-6 infection in HIV-infected asymptomatic and AIDS patients. *Intervirology* 1998; 41: 1–9.
24. Chan PKS, Peiris JSM, Yuen KY, *et al.* Human herpesvirus-6 and human herpesvirus-7 infections in bone marrow transplant recipients. *J Med Virol* 1997; 53: 295–305.
25. Fraisse P, Faller M, Rey D, *et al.* Recurrent varicella pneumonia complicating an endogenous reactivation of chickenpox in an HIV-infected adult patient. *Eur Respir J* 1998; 11: 776–778.
26. Ikuta K, Satoh Y, Hoshikawa Y, Sairenji T. Detection of Epstein-Barr virus in salivas and throat washings in healthy adults and children. *Microbes Infect* 2000; 2: 115–120.